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Review

Epicardial Origin of Resident Mesenchymal Stem Cells in the Adult Mammalian Heart

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Abstract: The discovery of stem and progenitor cells in the adult mammalian heart has added a vital dimension to the field of cardiac regeneration. Cardiac-resident stem cells are likely sequestered as reserve cells within myocardial niches during the course of embryonic cardiogenesis, although they may also be recruited from external sources, such as bone marrow. As we begin to understand the nature of cardiac-resident stem and progenitor cells using a variety of approaches, it is evident that they possess an identity embedded within their gene regulatory networks that favours cardiovascular lineage potential. In addition to contributing lineage descendants, cardiac stem cells may also be stress sensors, offering trophic cues to other cell types, including cardiomyocytes and vasculature cells, and likely other stem cells and immune cells, during adaptation and repair. This presents numerous possibilities for endogenous cardiac stem and progenitor cells to be used in cell therapies

or as targets in heart rejuvenation. In this review, we focus on the epicardium as an endogenous source of multi-potential mesenchymal progenitor cells in development and as a latent source of such progenitors in the adult. We track the origin and plasticity of the epicardium in embryos and adults in both homeostasis and disease. In this context, we ask whether directed activation of epicardium-derived progenitor cells might have therapeutic application.

Keywords: proepicardial organ; epicardium; mesenchymal stem cells; cardiac stem cells; cardiac fibroblast; CFU-F

1. Overview of Epicardial Development

The epicardium is the outermost mesothelial layer enveloping the heart in vertebrates, which has been long regarded as an inert structural layer or, at best, one that protects and lubricates the myocardium. However, mesothelial cells lining the body cavities are known to be metabolically active cells that can participate in homeostasis and responses to injury. They have a squamous epithelial morphology irrespective of their anatomical site and upon injury become cuboidal and adopt reactive states, seen by an abundance of microvilli and intracellular organelles [1]. They are active in immune surveillance, the control of inflammation and protection against tumour dissemination. Furthermore, in development, the mesothelia serve as a major source of progenitor cells for smooth muscle and interstitial fibroblasts for a range of visceral organs [2]. Mounting evidence attributes similar functions to the epicardium, with its demonstrated ability to contribute both multi-lineage descendants and trophic factors during cardiogenesis and cardiac repair [3,4] and to mount responses to pro-regenerative factors [5,6].

The epicardium originates from a cluster of extracardiac cells termed the proepicardium (PE) that condenses between the liver mesenchyme and sinus venosus of the forming heart tube at the reflection between myocardium and pericardium [7]. In chick, the PE is formed adjacent to the right sinus horn, and this right-sided localisation is determined by the left/right asymmetry pathway involving right-sided expression of transcription factors (TFs) Snai1 and Twist1 [7], both of which have been implicated in epithelial-to-mesenchymal transition (EMT), as well as induction and maintenance of stem cell states [8]. In the mouse, however, the PE is a midline structure. The liver bud may play a role in specification of the PE in chick, and during heart looping, the PE retains its proximity to liver mesenchyme as it comes to lie next to the atrio-ventricular (AV) canal [9]. In zebrafish, however, the liver is not an essential inductive source. Rather, Tbx5a determines competence for PE specification, and BMP4 originating from the heart tube activates TFs Tcf21 and Tbx18 in the PE; these pathways are essential for PE specification [10]. In chicken and potentially mammals, there appears to be an additional PE located at the arterial pole where the pericardium meets the outflow tract myocardium [11].

Genetic lineage tracing studies using Cre recombinase technology suggest that the PE arises within the early cardiac progenitor fields marked by the expression of cardiac TFs Nkx2-5 and Isl-1 [12]. From these and other findings [13,14], it would appear that the PE is an endogenous progenitor cell population specified within the cardiac fields at the juxtaposition between somatic pericardial

precursors and the splanchnic mesoderm that gives rise to myocardium. However, the PE is largely held over from differentiating into myocardium or vascular lineages until later in development. The PE has been proposed to be a vestigial remnant of the intermediate mesoderm in the anterior of the embryo, explaining its expression of nephrogenic genes, such as those encoding Wt1, podoplanin and Tcf21/capsulin [11]. Although Nkx2-5 expression does not persist in the PE, a memory of the initial cardiac field origin of the PE is potentially reflected in the transient expression of other cardiac TFs, including Tbx5, Tbx18, Tbx20, Gata4 and Srf, and the continuing expression of Tbx18, Tbx20 and Gata4 in the epicardial layer [15–17]. In *Nkx2-5* knockout mice, PE development is significantly impaired [12], while in embryos hypomorphic for Nkx2-5 [18], it is enlarged and overactive [19], suggesting significant, yet complex roles for Nkx2-5 in PE development. The PE is absent in Gata4 mutants [20], and in Tbx18 mutants, both epicardium and descendant coronary arteries are defective [21,22].

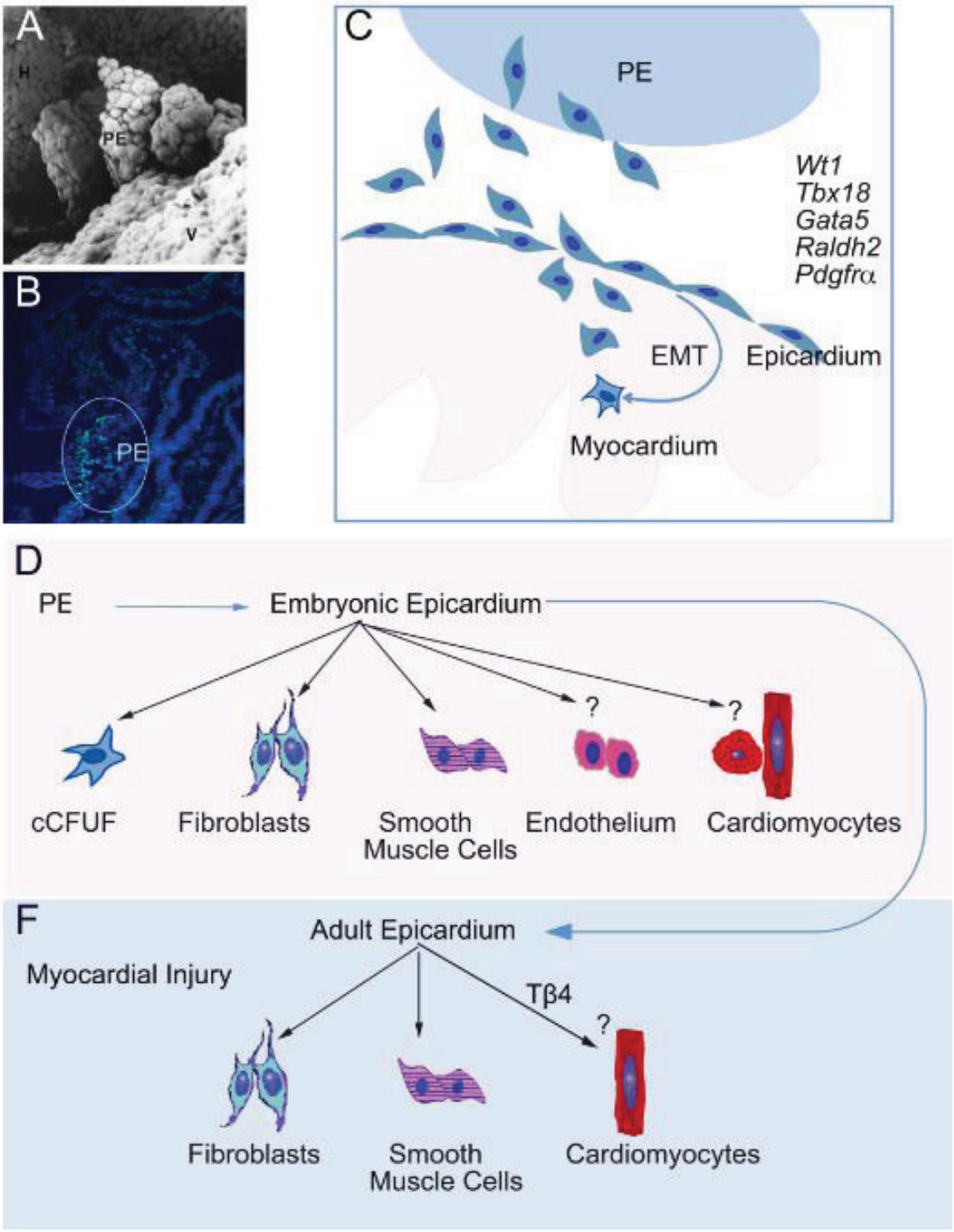
Structurally, the PE is composed of an outer epithelial layer convoluted into villi (Figure 1A,B), encompassing a core of mesenchymal cells that do not express canonical markers of the PE mesothelium, such as Tbx18, Wt1, Tcf21/capsulin and Cfc1 [9]. The core contains some endothelial cells that may have their origins in the chords of the endothelial plexus of the adjacent forming liver bud, involved in its specification [9,23]. After formation, the PE undergoes extensive proliferative expansion and migration, contributing the epicardial layer to the outer surface of the forming myocardium. Initiating this process, PE cells are either shed into the pericardial fluid, after which they alight on the myocardial surface as clumps or individual cells or are transferred by way of bridges of extracellular matrix at direct points of contact between the PE villi and myocardium (Figure 1C). Different vertebrate species likely utilize both of these mechanisms, although they may favour one. In the chick, which predominantly uses the bridging method, Tbx5 and BMP are involved, not only in specification [10], but also in regulating PE protrusion onto the myocardium [24,25]. Once PE cells contact and migrate over the myocardial surface, they expand to form the intact epicardial layer that progresses first around the atrioventricular furrow at the inner curvature, then further to cover the ventricles, atria and, finally, the outflow tract. It is noteworthy that epicardial outgrowth extends cranially to the region of the outflow tract that is later contained within the pericardium [26].

Physical inhibition of epicardial outgrowth in avian embryos or its genetic interruption in mice leads to abnormalities in heart looping, compact myocardial layer expansion, myocardialisation of endocardial cushion tissue and the septation and formation of coronary vasculature [27–29]. As discussed below, the developing epicardium, while directly contributing lineage components to the forming heart, also has major additional influences on heart morphogenesis through the production of trophic factors and the structural matrix.

2. Epicardial Plasticity in the Embryo

Across the atrial roof, the epicardium is positioned in close proximity to underlying myocardium. However, surrounding the rest of the heart, a clear subepicardial space is formed that is matrix-rich and populated by fibroblastoid cells [30]. These cells represent the earliest migratory derivatives of the epicardium.

Figure 1. Structure and fate of the proepicardium. (**A**) The proepicardium (PE) of a quail embryo (stage HH21) as seen by scanning electron microscopy. The villous nature of the PE in the phase of adhesion to the ventricle (V), whilst still adjacent to the hepatic surface (H), is evident (courtesy of Dr. Ramon Muñoz Chapuli) [26]. (**B**) The PE is marked by immunofluorescence for LacZ expression in an E8.5 embryo derived from crossing *Gata5*-Cre × Z/EG reporter mice. (**C**–**F**) Schematic illustrations of PEO cell migration and differentiation in embryonic and adult hearts after myocardial injury. EMT, epithelial-to-mesenchymal transition; cCFU-F, cardiac colony forming units-fibroblast.



The transit of cells from the PE to the epicardium and sub-epicardium involves changes in the epithelial state. Epithelial-to-mesenchymal transition (EMT) and its reverse, mesenchymal-to-epithelial transition (MET), occur in dynamic flux. Even as the epicardial layer adopts its squamous mesothelial form, some of its component cells retain mesenchymal characteristics and migrate into the subepicardial space, then subsequently into the myocardial interstitium, driven by EMT [31]. The multi-lineage potential of these so-called epicardium-derived cells (EPDC) as a population has been well established for over two decades. Dye labelling, viral tagging, cell transplantation, *in vitro* differentiation analysis and, more recently, genetic lineage tracking using Cre recombinase suggest that EPDCs contribute to components of coronary vasculature, interstitial fibroblasts/stromal cells and potentially cardiomyocytes during heart development (Figure 1D) [31–33].

There are, however, ongoing controversies surrounding precisely the breadth of lineages that derive from epicardium. The first relates to its contribution to the endothelium of the coronary arterial and venous vasculature. Recent Cre recombinase-based lineage tracing experiments suggest that the majority of coronary endothelial cells originate from the sinus venosus endothelium and/or the endocardium [34,35]. Studies using *Tbx18*-Cre, *Gata5*-Cre and *Tcf21*-merCremer as epicardially-restricted Cre drivers showed no contribution of epicardium to coronary endothelium [36–39]. However, Cre recombinase lineage fate mapping using *Wt1*-Cre as an epicardial Cre driver suggested that a minor population of endothelial cells were EPDC-derived. In a separate study, two independent epicardial-restricted Cre drivers, *Semaphorin 3D (Sema3D)*-Cre and *Scleraxis (Scx)*-Cre, tagged descendant lineages, including endothelial cells of both arterial and venous identity (24% labelling at postnatal Days 4–7 using the *Scx*-Cre), as well as coronary smooth muscle cells and fibroblasts, as expected [40]. With respect to the lineage findings using the *Wt1*-Cre line, endogenous expression of Wt1 RNA and protein has been detected in endocardium and coronary arteries, questioning the specificity of this Cre line for mapping endothelial fates of the PE [41,42].

The specificity of Cre drivers impacts on the important question of: what are the true lineage fates of the PE and epicardium? Due to the physical proximity of the PE epithelium to other vascular structures, in particular, the sinus venosus and liver plexus, the use of Cre lineage drivers that mark local vasculature or mesenchyme, as well as PE may lead to labelling of existing endothelial cells or their progenitors. Manual cell labelling techniques relying on DiI or viral labelling of cells, or cell transplantation, as used in earlier studies [31], may also lead to labelling of local mesenchyme and vasculature. During the early stages of PE induction, expression of Wt1, Tbx18 and *Sema3D* is detected in broad domains, encompassing both the lateral and intermediate mesoderm [9,40]. Perhaps already specified endothelial cells migrate along with true descendants of the PE mesothelium into the epicardial layer. Alternatively, as for the sinus venosus endothelium, they may track through the sub-epicardial space, thought to be one of the main routes for formation of the coronary venous sinus and arterial systems [34]. A further possibility is that the PE is composed of a mixture of progenitor cells with different potencies (see below). Increasingly, the specificity of Cre driver lines falls under closer scrutiny as the Cre technique is used to map the fate of stem and progenitor cell populations [43]. Heterogeneity of the cell lineage or cell state might impinge on the interpretation of Cre-lineage tracing experiments [40,44], and as suggested by Katz *et al.*, endothelial cells within (or surrounding) the PE may be labelled by only a subset of Cre drivers [40].

The contribution of EPDCs to cardiomyocytes [36,37] is also disputed. Studies using quail-chick chimeras or retroviral lineage tracing reported no evidence of EPDC-derived cardiac muscle cells [29,45]. However, PE explants readily differentiate into cardiomyocytes under the influence of bone morphogenetic protein [46]. Furthermore, fate map studies using *Wt1*-Cre and *Tbx18*-Cre suggested a significant contribution of epicardium to cardiomyocytes (in the study using *Wt1*-Cre, 4–18% of total cardiomyocytes in the foetal heart were scored as epicardium-derived depending on the heart chamber and method of quantification) [36,37]. Fate mapping using *Sema3D*-Cre and *Scx*-Cre also showed a small contribution of EPDCs to cardiomyocytes (up to 6.6% of total cardiomyocyte at postnatal Days 4–7 scored as epicardium-derived using *Scx*-Cre) [40], and a myocardial contribution was observed after priming of the heart with the vasoactive molecule thymosin beta4 in the context of myocardial infarction (MI) and *Wt1*-Cre lineage tracing (6.2% of cardiomyocytes in the peri-infarct region scored as epicardium-derived) [5,47] (see below). Both Wt1 and Tbx18 alleles, however, show ectopic or endogenous expression in cardiac muscle; the *Wt1*Cre-EGFP allele in particular was found to be expressed in a stochastic manner throughout the entire embryo, including in cardiomyocytes [41], at least one day earlier than endogenous Wt1 protein expression in PE, and this ectopic signal may derive from transient expression of Wt1 during gastrulation [48]. A potentially related finding is that the *Gata5*-Cre transgenic allele, which marks epicardium, also shows stochastic expression in myocardium from before epicardial deployment [49]. Tbx18 was found to be normally expressed by a minor population of cardiomyocytes in the left ventricle and interventricular septum, in addition to epicardium [17]. Later studies using a different *Tbx18*-Cre driver and a *mesothelin* (*Msln*)-Cre driver (from P0) showed that epicardium contributes fibroblasts and smooth muscle cells, but not cardiomyocytes to the heart [2,50]. This issue remains unresolved, and definitive answers to the question of whether the epicardium contributes to coronary endothelial and cardiomyogenic lineages await the development of more specific and single-cell-based lineage tracing approaches.

These discrepancies notwithstanding, the ability of the embryonic epicardium to generate cells of the smooth muscle and fibroblast/stromal lineages is now accepted. An important question is whether the PE and epicardium contain cells that have multi-lineage potential or a collection of several lineage-restricted subpopulations reflecting epicardial heterogeneity. As noted above, the most recent experiments addressing the multipotency of epicardial cells have come from genetic lineage tracing studies using Cre recombinase [36,37,51], while more classical approaches have used heterologous tissue transplantation [52] or targeted cell engraftment [53]. All of these approaches suffer from a lack of single cell resolution. One study in avian embryos did approach lineage tracing at single-cell resolution using low-titre replication-incompetent retrovirus carrying a *β-galactosidase* cassette to indelibly tag cells [54]. Labelling of mesocardium at embryonic Stage 15 or earlier exclusively labelled myocardium at later stages. Mesocardial labelling at Stage 17 or later, however, resulted in distinct clones with unipotent differentiation potential (myocytes, coronary endothelial cells, perivascular fibroblasts or vascular smooth muscle cells). Here, the authors suggested that an extra-cardiac source of cells entering the heart was labelled by the virus and contributed cells to vascular and other cardiac lineages. Furthermore, if a common multipotent progenitor gives rise to all or some of the traced cell types, its lineages must have been resolved before migration of those cells into the heart. A later study in which direct viral tagging of the PE was performed confirmed the general conclusion that only unipotent cells populate the PE [23].

3. Adult Cardiac Stem/Progenitor Cells

The discovery of stem-like cells in adult hearts has provided a vital dimension to cardiac regeneration. In adult hearts, cells marked by the expression of surface proteins found on other stem cell types, including the tyrosine kinase receptor, cKit [55], TF Isl1 [56], stem cell antigen Sca1 [57] or PDGFRα [49], have been identified. A number of stem cell-intrinsic properties have also been used to identify or validate potential adult cardiac stem cell populations, such as the efflux of toxic compounds by transporters (side population) [58], the ability of cells to retain nucleotide analogues (long-term label retention), which reflects their slow cycling [59], or colony formation [49]. In some cases, such cells have been demonstrated to possess long-term growth and clonal multi-lineage differentiation potential *in vitro* or *in vivo* (in the context of transplantation), hallmarks of stem cells. Indeed, based on the expectation that adult cardiac stem and progenitor cells carry a differentiative bias toward cardiomyocytes, as well as coronary vascular lineages, cell therapy clinical trials have been initiated using expanded cKit+ cardiac progenitors or adherent cells cultured from cardiac non-adherent spheroids (cardiospheres) in patients with ischaemic cardiomyopathy [60,61]. The relationships between the different populations discovered and their origins are poorly understood, and it is likely that some represent different states in a single cellular hierarchy. The use of markers alone is a highly unreliable approach to establishing lineage relationships. The issue of stem cell origins is complicated by the influx of bone marrow cells expressing stem cell markers (including cKit) after myocardial infarction [62]. Nonetheless, recent evidence derived from the visualization of the expression of a cKit-GFP transgene suggests that a population of adult cardiac progenitors bearing cell-surface cKit has its origins in a cardiomyocyte interstitial niche established early during development before deployment of the epicardium [63,64].

For the remainder of this review, however, we focus on the embryonic epicardium as a source of mesenchymal stem and progenitor cells that persist into adulthood and ask whether reactivation of embryonic epicardial gene programs can reset the cardiogenic memory of adult epicardium.

4. Epicardium-Derived Stem/Progenitor Cells

Our group has previously described a population of colony-forming cells that reside within the cardiomyocyte interstitium and adventitia of coronary arteries in young adult mouse hearts [49]. These cells form mesenchymal stem/stromal cell (MSC)-like cultures akin to those originally described in BM [65,66]. The BM MSC concept, as modified over the years [67], describes multipotent self-renewing stem cells that reside in the perisinusoidal compartment of BM and which give rise to a variety of skeletal connective tissue types, including boney parenchyma, cartilage, adipocytes, fibroblasts and stromal cells capable of sustaining hemopoiesis. Fate mapping studies in the context of transplantation have now established this hierarchy at a single-cell level [68]. Studies in embryonic stem (ES) cells suggest that MSCs represent a persistent early embryonic mesodermal cell subtype derivative of precursors with both mesenchymal and endothelial potential [69]. The MSC concept is rooted in studies dating to the 1960s on the skeletogenic properties of BM stroma when transplanted ectopically [70], and over the ensuing decades, it has generated intense interest worldwide, not only in the underlying biology, but also for the development of cell-based off-the-shelf therapies for diverse disease states.

There are in fact over 300 clinical trials using bone marrow or other sources of MSCs in progress. Controversies have arisen surrounding a number of points: the generalisation of the BM MSC concept to all tissues; their generic equivalence to pericytes; the meaning of the apparent broad potency of MSCs revealed by *in vitro* assays and associated claims of pluripotency; and the scientific justification for their use in cell-based therapies for diseases of non-skeletal origin [67]. These issues continue to plague the field and, to an uncomfortable degree, overshadow the interesting biology that underpins the MSC concept and its importance for homeostasis and repair of a range of organs and soft tissues.

In the heart, we refer to colony founder cells as cardiac colony forming units-fibroblast (cCFU-F), in deference to the pioneering work of Friedenstein and colleagues on BM CFU-F and to avoid the use of the MSC tag to refer specifically to colony-forming founder cells *in vivo*, a nomenclature that has generated much consternation (see the review by Bianco *et al.* [67]). As in BM, colonies derived from cCFU-F differ in their *in vitro* lineage growth and potency. Some larger colonies (Figure 2G) show secondary colony formation in clonal assays and have the capacity for long-term growth [49]. Smaller colonies also have secondary re-plating ability and, while they can generate large colonies, do so in a diminishing fashion and have only limited *in vitro* growth potential. The smallest colonies (microcolonies) do not re-plate and appear differentiated to smooth muscle or myofibroblast-like cells. The colony assay, therefore, would appear to capture a spectrum of cell states in relation to growth and clonogenicity. Likewise, cells within MSC-like cultures are not equivalent when re-plated, with a minority fraction having secondary colony forming ability and some differentiating into myofibroblast-like cells. Thus, cardiac MSC-like colonies, grown typically on tissue culture plastic in high serum, represent a metastable system. While the *in vitro* readouts of long-term growth and lineage potency are potentially valuable surrogates for the intrinsic stem cell states of self-renewal and plasticity, they are in themselves problematic, since, unlike for blood, which generates billions of cells daily from a rare population of slow cycling stem cells and transit amplifying progenitor cells, the heart is an organ with slow homeostatic turnover. Thus, exactly how long-term *in vitro* growth relates to the stem character of cCFU-F *in vivo* is still unknown. Likewise, lineage potency, as determined *in vitro* using strong biochemical drivers and limited markers and functional readouts, almost certainly reflects more the suggestibility of the cell's gene regulatory networks to reprogramming than its lineage fate *in vivo* (see below). Understanding the system fully can only proceed with robust *in vivo* assays of cell state, behaviour and fate. Nonetheless, with these caveats clearly in mind, *in vitro* colony assays have provided an essential comparative framework for probing the qualities of a range of rare embryonic and adult stem cell populations and a template for interrogating biochemical pathways and network dynamics underpinning stem and progenitor cell biology.

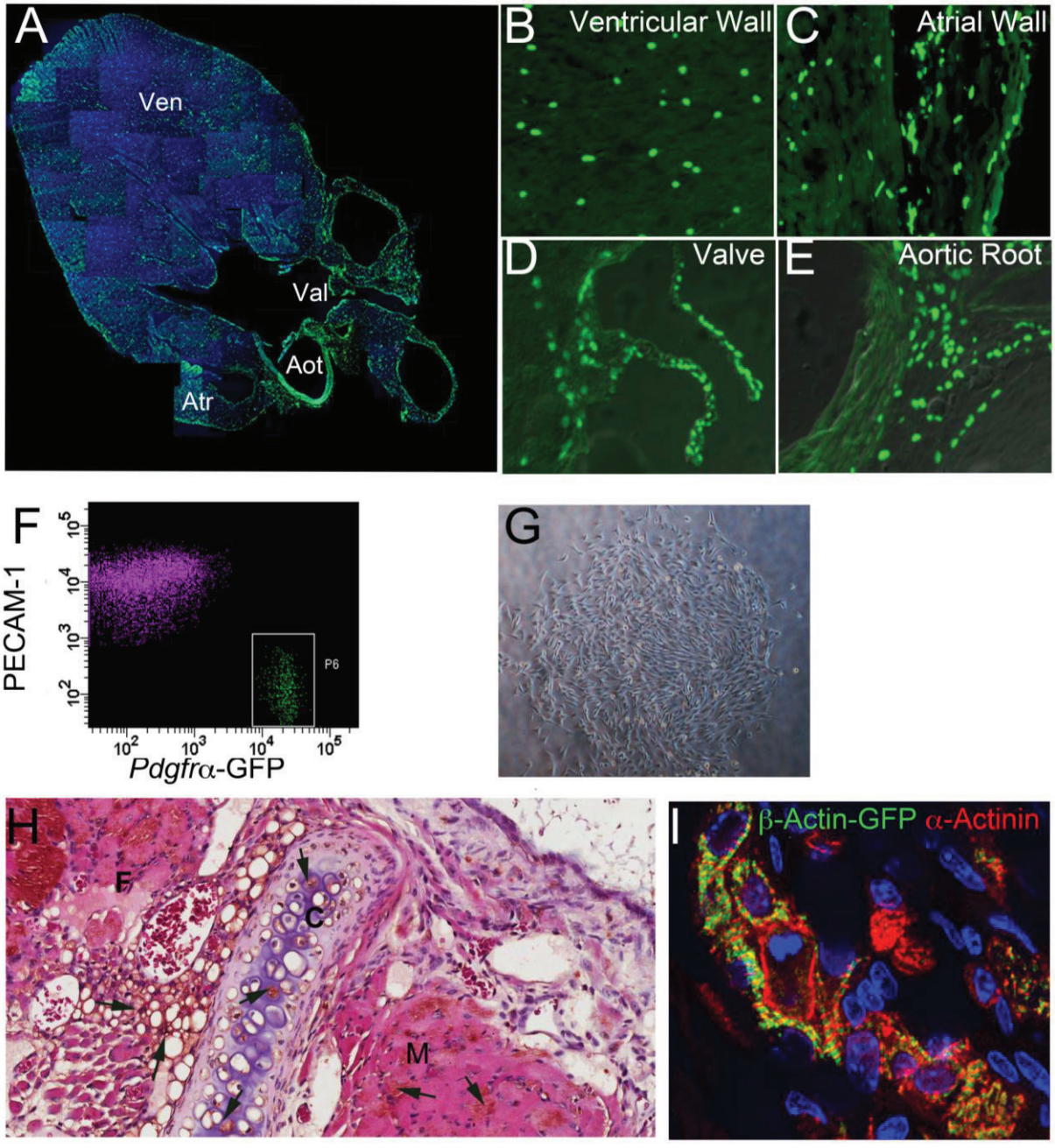
The similar (although not identical) transcriptome and cell surface marker profiles of bulk cultures of cCFU-F colonies derived from mouse heart, kidney and BM [66], including the expression of "canonical" MSC markers, such as Sca1 (rodents), CD44, CD105 and CD90, suggest that organ-specific CFU-F share a common evolutionary origin and potential functionality, without implying equivalence. We have conducted BM transplantation and Cre recombinase-based lineage tracing experiment to address: (1) the lineage origin of cardiac CFU-F in development; and (2) their potential equivalence to BM CFU-F from the perspective of lineage origins in ontogeny. These studies have revealed a number of relevant findings. BM transplantation using GFP-tagged donor marrow and lead shielding of the heart to protect against radiation damage to the CFU-F niche showed that cCFU-F giving rise to large

colonies are not in significant flux with BM cells, including BM CFU-F, which readily engraft in this assay, for the greater part of the adult life of a mouse. This also holds true after myocardial infarction, during which abundant immune and reparative cells are mobilized from BM to the site of ischaemic injury [49,62] and after mobilisation of blood stem cells into the circulation using the hormone, GCSF [49]. cCFU-F appear distinct from the extremely rare cKit$^+$ cells present in healthy adult hearts and from the abundant cKit+/CD45+ cells that flood in from BM after MI.

Cre lineage tracking using a variety of Cre drivers showed that cCFU-F in the embryonic (E17.5) and young adult mouse hearts are largely, if not exclusively, from the mesoderm, but do not derive from neural crest or endoderm or from de-differentiation of cardiomyocytes. Tracking with *Gata5*-Cre and *Wt1*-CreERT2 drivers, their limitations notwithstanding, strongly suggests that cCFU-F have their origins in the epicardium during development. Activation of Cre from the conditional *Wt1*-CreERT2 driver is tamoxifen-dependent, and while this modification renders lineage tagging less efficient, a significant proportion of cCFU-F colonies were tagged after a tamoxifen induction regime that avoided the possibility of early spurious embryo-wide induction seen for a separate non-conditional Wt1-Cre transgene. While these Cre drivers are not perfectly specific for the epicardium or PE (see above), in combination with other Cre drivers that exclude a muscle and neural crest origin, an epicardial/PE origin seems most likely. Our analysis does not exclude a minor contribution from other lineages or that in different growth conditions (e.g., matrix, serum), other cell types with distinct origins may be captured by the colony assay.

The conclusion of an epicardial origin for cCFU-F is supported by studies in the embryo. A valuable marker that allows enrichment for cCFU-F by flow cytometry is the cell surface protein PDGF receptor alpha (PDGFRa) and, in particular, a knockin allele expressing nuclear GFP (*Pdgfrα*-GFP) (Figure 2A–E) [71]. In the adult heart, *Pdgfrα*-GFP+ cells are located within the coronary arterial adventitia, as also found in the ascending aorta (Figure 2E), and more broadly within the cardiomyocyte interstitium (Figure 2A–C) close to or in contact with the basement membrane of microvessels. *Pdgfrα*-GFP+ cells are the majority non-vascular cell type at this location, making PDGFRa a marker of the population generically referred to as cardiac fibroblasts. While these cells are perivascular in location, they are never ensheathed by the collagen IV+ basement membrane of endothelial cells, as would be the case for true pericytes [72]. In healthy hearts, cCFU-F is captured entirely by the *Pdgfrα*-GFP+/Sca1+/CD31− non-myocyte fraction, which is easily isolated as a distinct fraction by flow cytometry (Figure 2F). The cardiac valves and annulus fibrosus are also replete with *Pdgfrα*-GFP+ cells (Figure 2D), although these bear few CFU-F, if any. The adult epicardium contains some *Pdgfrα*-GFP+ cells (~5%), but importantly, these do not have CFU-F activity after isolation by flow cytometry using a pan-cadherin antibody.

Figure 2. Platelet-derived growth factor receptor alpha is a marker of cardiac interstitial and perivascular fibroblasts and cardiac colony forming units-fibroblast (cCFU-F). (**A**–**E**) Cells exhibiting nuclear GFP fluorescence in *Pdgfrα*-GFP knockin mice are distributed throughout the myocardial interstitium, valve leaflets and aortic root. Cardiac CFU-F cells are captured among the Sca1+/*Pdgfrα*-GFP+/PECAM1− fraction on flow cytometry. (**F**) The figure shows the Sca1+ fraction segmented using GFP and PECAM1 antibody. (**G**) A representative large colony derived from the cardiac CFU-F fraction. (**H**, **I**) When cardiac mesenchymal stem/stromal cell (MSC)-like cultures are implanted under the kidney capsule with embryonic stem cells, they survive and differentiate into a range of mesodermal, endodermal and ectodermal lineages. Panels show fat (F), chondrocytes (C) and skeletal muscle (M) (brown histochemical staining for GFP, which marks cardiac MSC-derived cells, arrows) and cardiac muscle (GFP and cardiac alpha actinin immunostaining).



In post-gastrulation development, *Pdgfrα*-GFP is expressed broadly in mesoderm and neural crest. In the heart, it marks the PE and surrounding mesenchyme, then the developing epicardium and EPDCs. PDGFRa is also expressed in myocardial progenitor cells of the heart fields, although it turns off when these cell differentiate into muscle [18]. Thus, PDGFRa is a highly specific developmental marker for epicardium and, potentially, its EPDCs. In fact, one can observe the apparent movement of *Pdgfrα*-GFP+ cells from the epicardium into the sub-epicardial space and then into the cardiomyocyte interstitium, acknowledging that following marker expression is not a formal method of lineage tracing. In the interstitium, *Pdgfrα*-GFP+ cells associate closely with CD31+ endothelial cells of the coronary circulation and do not express the cardiomyocyte TF, Nkx2-5. Apart from its role as a marker of cCFU-F, PDGFRa is a functional component of epicardial EMT acting, in particular, upstream of the cardiac fibroblast compartment [73]. Loss of *Pdgfrα* in mice causes severe defects in epicardial EMT, with the loss of the TF, Sox9. Cardiac fibroblasts marked by CD90/Thy-1 are diminished with reduced transcript levels for fibroblast-enriched genes, including type 1 collagens (Col1a1, Col1a3), discoidin domain receptor 2 (DDR2) and periostin. Although smooth muscle did not appear to be affected in *Pdgfrα* mutants, in our studies, *Pdgfrα*-GFP^{low} cells were observed in the developing smooth muscle of forming coronaries and the aorta [49], suggesting their origin from the *Pdgfrα*-GFP+ population and consistent with research on epicardial lineage fate (see above). We have tracked cCFU-F activity back through foetal development and confirmed enrichment exclusively in the *Pdgfrα*-GFP+ cell fraction, and CFU-F activity is observed even within cells micro-dissected from the PE. Furthermore, large CFU-F activity is severely diminished in foetal hearts from *Pdgfrα* hypomorphic mutant mice [74], suggesting a vital role for PDGF signalling in the maintenance of cCFU-F.

5. Lineage Potency of Cardiac MSC-Like Cells

We have explored the lineage potency of bulk-cultured cCFU-F in a variety of *in vitro* and *in vivo* assays. Using *in vitro* assays routinely employed to assess differentiation to different lineage outcomes, clonally-derived cCFU-F cultures showed a strong propensity for the formation of cardiomyocytes, smooth muscle, endothelial cells, adipocytes, chondrocytes and osteocytes, as judged by the expression of multiple markers and even to liver endoderm and, to a lesser extent, neural lineages, suggesting a trans-germ layer outcome. Endothelial cells were capable of acetylated low-density lipoprotein uptake and formed branched vascular-like networks in Matrigel *in vitro*, while smooth muscle cells contracted in the presence of the muscarinic receptor agonist carbachol. The freshly isolated *Pdgfrα*-GFP+/Sca1+/CD31− cell fraction also showed a similar differentiation profile, albeit with a lower probability of lineage conversion, suggesting that this type of outcome is not an artefact of cell expansion in culture and not restricted to the progeny of CFU-F. As noted above, such assays should not be interpreted to indicate the normal lineage fate of cells *in vivo*, rather an open state of chromatin, allowing them to be readily directed along certain lineage fates in response to extracellular chemical signals; as has been argued, a form of reprogramming [67].

We devised an alternative assay to assess the ability of these cells to be directed by extrinsic signals in a transplantation environment. Injected under the kidney capsule, bulk-cultured cCFU-F did not survive well or form teratomas, indicating that they are not pluripotent in the accepted definition, as

applied to ES cells. However, when co-transplanted with ES cells, GFP-tagged cultured cCFU-F cells were able to survive within the resultant ES cell-derived teratomas and to contribute to a variety of mesodermal, endodermal and ectodermal lineages, as judged by morphological outcomes (Figure 2H). This included immature cardiomyocytes bearing alpha-actinin+ striated sarcomeres (Figure 2I). Bulk-cultured cCFU-F cells could also form occasional cells resembling striated cardiomyocytes, as well as vascular endothelial and mural cells when injected into the surviving myocardium of infarcted hearts, albeit that this hostile environment does not favour the survival of transplanted cells. We interpret these results to indicate that cCFU-F, their *in vitro* culture descendants and the potentially related "fibroblast" populations *in vivo* are immature stem or progenitor cells that are highly impressionable in the face of a variety of extracellular signals, directing them to engage at least the first stages of the lineage differentiation pathways. This implies that their lineage fates *in vivo* are not highly canalized or cell-autonomous. This would seem intuitive for this variety of adult stem cell, which may be called upon to respond to a variety of different natural and hostile stimuli to effect both adaptation and repair. Their *in vivo* fate remains to be determined; however, drawing on what the field has learned from the BM CFU-F system, we anticipate that cCFU-F are endogenous cardiac MSC-like stem cells, whose principal lineage function is to renew the population of resting fibroblasts in the heart during development, homeostasis and repair. These cells likely represent uni- or multi-potent progenitor cells, retaining an open chromatin state that allows them to respond to a diversity of cues and differentiate into myofibroblasts, smooth muscle, fat and other specialized stromal and connective tissues.

Such cells likely have many other functions as sensors of stress, providing paracrine or niche support to other stem cells and vascular elements, as well as cardiomyocytes [75,76]. The cCFU-F lineage doubtless also contributes to pathological fibrosis in hypertension and other cardiac pathologies and the fibro-fatty infiltration that accompanies certain myopathies [77]. Fibro-calcification of the pericardium and cardiac valves may also involve CFU-F/MSC-like cells [78]. This view of the cCFU-F lineage hierarchy requires rigorous testing. It is unlikely that the cCFU-F hierarchy forms cardiomyocytes in significant numbers during development or in adulthood. However, the possibility for this outcome raised by the teratoma co-culture experiments discussed above suggests that this fate outcome is normally repressed. Recent experiments on direct cellular reprogramming of cardiac fibroblasts to cardiomyocytes *in vivo* [79] likely draw on the inherent cardiac identity of these cells and the ability to de-repress a cardiomyogenic outcome. This is clearly interesting from a therapeutic perspective, and deeper analysis of the networks underpinning cCFU-F identity and function will be fruitful.

6. The Adult Epicardium as a Regenerative Platform

Although adult epicardium is generally assumed to be quiescent, there is evidence for latent epicardial activity. Maintenance of functional and structural integrity of adult zebrafish heart is, to an extent, due to the continuous contribution of epicardial cells to the ventricular wall [80]. Manual DiI labelling of the zebrafish epicardium, as well as genetic lineage tracing using *Tbx18*-Cre show the robust contribution of epicardial cells to the growing ventricular wall in adult zebrafish in an FGF-dependent manner. Adult epicardium and the subepicardial space represent a hypoxic microenvironment [81]. Low metabolic activity and low oxygen domains were mainly identified along

ventricular sub-epicardium populated with cells that expressed the hypoxia inducible factor 1-alpha (HIF1α), which becomes stabilized under hypoxic conditions. These cells were shown to adopt cytoplasmic glycolysis over mitochondrial metabolism, which is often the protective metabolism of choice for stem/progenitor cells against reactive oxygen species (ROS). They also expressed markers of cardiac progenitors (Nkx2.5 and Gata4), and these cells had the ability to differentiate to cardiogenic lineages [81]. Cardiac stem-like cells were also located by electron microscopy in the epicardial niche, being small (6–10 um), round cells with an irregular-shaped nucleus, large nucleolus, few endoplasmic reticulum cisternae and mitochondria, but numerous ribosomes. These cells were suggested as a regenerative core in the adult mouse heart [82]. These conclusions are, however, merely based on morphological and electron micrographical observations. *In vitro* evidence also supports epicardial reactivation. In culture, human adult epicardial explants undergo spontaneous (or TGF-β-stimulated) EMT [83] and differentiate into smooth muscle cells [84]. These explants were also shown to differentiate into cells expressing the adult stem cell epitope, cKit [85].

Although healthy adult epicardium seems to have the potential to reactivate its developmental program, this is more prominent in response to cardiac injury. Zebrafish hearts can regenerate after resection of the ventricular apex, mainly through dedifferentiation and proliferation of existing cardiomyocytes, which are in parallel accompanied by a global activation of the epicardium [3]. Soon after ventricular resection, expression of *Raldh2, Tcf21* and *Tbx18*, components of the embryonic epicardial program, was visible within the entire epicardium [51]. After a few days (by 14 days post amputation), the epicardial response localized to the wound area, with cells actively invading the newly forming muscle, contributing cells to the new vasculature. *In vivo* knockdown studies show an essential role for epicardial Fgf receptors and Raldh2 in the injury response [3,51]. As in zebrafish, in mouse hearts, myocardial infarction (MI) leads to proliferation and expansion of the existing epicardium and reactivation of its foetal program, including expression of several epicardial transcription factors, like Wt1 and Tbx18 [4,5]. Further genetic or viral lineage tracing demonstrated that these activated epicardial cells differentiate into the smooth muscle component of new vessels, as well as into fibroblasts and myofibroblasts, although not cardiomyocytes or endothelial cells [49,86]. These *in vivo* studies powerfully reinforce the central role played by epicardium in heart regeneration.

In humans, epicardial cells were found missing from the surface of hearts with ischemic cardiomyopathy, suggesting epicardial migration post-ischemia [85]. Furthermore, rare stem-like cells expressing cKit were localized within the adult epicardium, which, after MI, tend to proliferate, enter the sub-epicardial space and express the cardiac transcription factor, Gata4. $cKit^+$ cells were later found in the smooth muscle and endothelial compartments of sub-epicardial vessels, suggesting a possible functional/structural role for these cells as cellular components of the vessels [85,87].

The ground state of epicardial activation can be further triggered by ectopic signals that can either enhance the existing levels of activity, or potentially mediate the transition of epicardial progenitors into cardiogenic fates. The 43-amino acid actin monomer-binding peptide, thymosin beta 4 (Tβ4), has recently attracted a lot of attention, due to its effect in promoting cardiac repair. In this context, Tβ4 was shown to reduce cardiomyocyte apoptosis and to promote cardiac function recovery in a murine MI model [47]. Furthermore, priming of hearts with Tβ4 before cardiac injury tends to expand the epicardium and promote its foetal program, as well as stimulating epicardial mobilization and differentiation into fibroblasts, endothelial and smooth muscle cells [5,88], as well as a minority of

cardiomyocytes [5]. The effects of this molecule in reprogramming epicardial fate, however, did not seem to be recapitulated, when Tβ4 was administered during MI [89]. The treatment of murine hearts with Tβ4 during and after myocardial infarction did lead to the expansion of the epicardium and enhanced cardiac injury repair, as mentioned above. Mobilization of the epicardial cells and/or differentiation of adult EPDCs into endothelium and cardiomyocytes, however, were not observed. Given that cardiac priming, as a therapeutic approach, is hardly feasible, the downstream mechanisms of Tβ4 action after treatment at different times should be compared, and this may possibly lead to the identification of additional factor(s) that could facilitate Tβ4-mediated reprogramming of adult epicardial cells.

Among the myriad of signalling pathways that are induced during cardiac injury, a notch signalling receptor-mediated injury response was also suggested in the adult epicardium [90]. Both thoracic aortic banding and myocardial infarction induced notch activation in a subset of murine epicardial cells, further shown to be highly enriched in fibrosis repair gene programs, with a potential towards fibroblast differentiation. Around 50% of these so-called notch-activated epicardium-derived cells (NEC) expressing MSC markers, including Sca-1 and CD44, are positive for the TGFβ receptor complex and lack CD34 and cKit expression. Furthermore, NECs grew clonally into heterogeneous cultures *in vitro* and showed modest cardiomyogenic potential when co-cultured with rat neonatal cardiomyocytes. Differentiated NECs showed up to a six-fold increase in cardiac muscle-specific transcripts, including cardiac *α-actinin*, *myomesin-1* and *troponin-C*. Differentiated NECs retained their *α-actinin* expression when transplanted into immune-compromised non-injured mouse hearts. The aspects of potency in these cells, however, still await more detailed analysis.

Finally, epicardial cells likely play a significant role in cardiac repair by the secretion of angiogenic and other paracrine factors after cardiac injury. In a detailed study [4], conditioned medium harvested from cultured adult EPDCs present after injury stimulated the proliferation of endothelial cells, and when these EPDCs were co-cultured with endothelial cells or injected *in vivo*, they incorporated into the vascular structures in the perivascular compartment. A large number of pro-angiogenic factors were found to be secreted by adult EPDCs after injury, including VEGF, angiopoietins, FGFs, PDGFs, SDF1 and MCP1. Injection of EPDC conditioned medium into the border zone immediately after induced myocardial infarction, reduced the infarct size and improved cardiac function at 5–6 days after injury, although functional improvements were not sustained.

7. Conclusion

During heart development, the epicardium shows multi-lineage potential as a population and displays highly dynamic behaviours, including EMT, growth and expression of trophic factors. As the embryo matures, these activities decline to basal levels. However, cCFU-F-cardiac resident epicardium-derived stem-like cells that retain the properties of foetal EPDCs and a memory of their cardiac origin-persist in the interstitium, while the epicardium itself retains latent activities associated with foetal epicardium. One must be cautious in drawing conclusions about the cellular state after *in vitro* culture, and it is therefore crucial that many of the conclusions and predictions concerning epicardium and cCFU-F be confirmed *in vivo*, for example by detailed lineage tracing of single cells. Nevertheless, data suggest that the epicardium exists in a primed state that likely plays major roles in

maintaining cardiac architecture during homeostasis, contributing to repair in the presence of acute injury-associated signals. Injury stimuli that persist during chronic disease lead to aberrant activities underpinned by excessive fibroblast and adipocyte differentiation.

A key component of epicardial activity in development and injury is the induction of EMT, a fundamental process in cell biology that can simultaneously initiate epicardial cell migration and impose mesenchymal identity on an otherwise dormant epithelium. Increasing evidence links EMT to core stem cell regulatory networks [8]. We could anticipate, therefore, that induction of EMT in epicardium and activation of cCFU-F could not only initiate cell migration and lineage deployment, but also reactivate stem/progenitor networks that enhance or further sustain the capacity for tissue repair. From this perspective, the identification of inductive signals that rejuvenate foetal programs in epicardium and cCFU-F after heart injury could lead to promising new approaches to the regeneration of damaged heart tissue, although much work needs to be done on the basic biology of epicardium, cCFU-F and EPDCs and how they maintain and access different cellular states.

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Author Contributions

Naisana Asli co-wrote the manuscript. Munira Xaymardan co-wrote the manuscript and prepared figures. Richard Harvey co-wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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